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Effect of Adding Green Tea Polyphenols to Bali Bull Spermatozoa Diluent: Implications for Semen Quality, Hydrogen Peroxide Concentration, and Fertilization Rate

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Green Tea Polyphenols (GTP) are bioactive compounds found in green tea and are known to have strong antioxidant properties. This study aims to determine the effectiveness of adding GTP in the extender in terms of quality, hydrogen peroxide (H_2O_2) concentration, and fertilization rate of spermatozoa *in vitro*. Semen samples were collected from Bali bull using the artificial vagina method. The collected semen was diluted with treatments without the addition of GTP (T0), T1 (0.01 mg/100 mL), T2 (0.02 mg/100 mL), and T3 (0.03 mg/100 mL) GTP with 5 repetitions. The parameters tested were motility viability, Acrosome integrity, Plasma membrane integrity, DNA fragmentation, fertilization rate, spermatozoa H_2O_2 concentration, and oocyte H_2O_2 concentration. The research results show that the addition of GTP to semen diluent significantly affects viability, acrosome integrity, plasma membrane integrity, DNA fragmentation, and concentration of H_2O_2 ($P<0.05$). It does not affect motility and sperm abnormalities ($P>0.05$). Adding GTP also significantly affects the fertilization rate of oocytes and the concentration of H_2O_2 in Bali cattle oocytes ($P<0.05$). Adding 0.02 mg/100mL GTP to the extender yielded the best results in improving sperm quality and fertilization ability in vitro. The study's conclusion on adding GTP to the extender provides positive implications for semen quality, reduces H_2O_2, and improves spermatozoa fertility.
Keywords: Beef cattle, artificial insemination, genetic improvement, cryopreservation, semen quality, ROS.

INTRODUCTION

Bali cattle are a type of beef cattle originating from Indonesia. One of the efforts to increase the productivity of Bali cattle is through the Artificial Insemination (AI) method. In district Woha, Bima, the percentage of successful artificial insemination was found in several types of cattle such as Simmental cattle (27.8%), Angus cattle (22.2%), Brahman cattle (17.7%), Bali cattle (16.7%), and Limousin cattle (15.6%) (Lukman et al., 2022). Artificial insemination is beneficial for genetic improvement and increasing the cattle population in Indonesia (Prihantoko et al., 2022). This method has become the primary method in developing genetically superior Bali cattle (Sonjaya et al., 2021). Efforts to support artificial insemination include the availability of high-quality frozen semen. Cryopreserving semen is prone to damage to spermatozoa cells, resulting in decreased quality. During cryopreservation, cells can experience much damage, such as osmotic dehydration, cold shock, and oxidative damage due to Reactive Oxygen Species (ROS) (Liu et al., 2021).
Spermatozoa damage due to ROS occurs through oxidative attack on sperm membrane lipids, triggering lipid peroxidation (Gualtieri et al., 2021). Lipid peroxidation caused by hydrogen peroxide (H_2O_2) not only disrupts sperm motility but also damages all sperm functions that depend on plasma membrane integrity, including sperm-oocyte fusion and the ability to undergo acrosome exocytosis (Bansal and Bilaspuri, 2010). Based on the results of the meta-analysis by Agarwal et al. (2005), it was concluded that there is a significant correlation between ROS levels in spermatozoa and the success rate of fertilization after in vitro fertilization (IVF) procedures.

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Lipid peroxidation induced by hydrogen peroxide (H_2O_2) has been proven to significantly impact sperm function (Bollwein and Bittner, 2018). Lipid peroxidation not only reduces sperm motility but also damages various sperm functions, such as the ability of sperm to fertilize oocytes and perform acrosome exocytosis, both of which are crucial in the fertilization process (Kaltsas, 2023; Bansal and Bilaspuri, 2010). Furthermore, oxidative stress caused by free radicals not only reduces the sperm's ability to fertilize oocytes both *in vitro* and *in vivo*, but also potentially hinders zygote development, leading to chromosomal abnormalities (Younus et al., 2024). The quality and fertilization capability of spermatozoa can be improved by adding various motility agents or antioxidants to the extender (Petel et al., 2016; Altyeb et al., 2022). Antioxidants have been used to protect spermatozoa from the effects of cryopreservation (Anghel et al., 2010). The polyphenols contained in green tea are a source of natural antioxidants that are effective in capturing reactive oxygen species (ROS). Green Tea Polyphenols (GTP) are the main components of green tea that are soluble in water and consist of various compounds, including flavonoids, flavanols, phenolic acids, and the like (Wang et al., 2007). Polyphenols in green tea work by directly reacting with ROS, forming relatively stable phenolic oxygen radicals, thereby eliminating free radicals that can damage cells (Yan et al., 2020). One of the main groups of polyphenols in green tea is catechins, which are part of flavonoids (Musial et al., 2020). Several studies on GTP have been previously reported by Susilowati et al. (2022) which can improve the quality of Kacang Buck semen during equilibration and post-thawing, Ongole Bull crossbreeds at frozen storage (Pramesti & Ducha, 2023) and can improve semen quality and reduce DNA mutations in buck sperm (Mustofa et al., 2021). This is the reason why the current research is focused on the addition of GTP on the extender to evaluate the quality of Bali cattle spermatozoa, measuring the concentration of hydrogen peroxide and the fertilization ability of spermatozoa.

MATERIALS AND METHODS

***Research location*:** This research was conducted at the Regional Artificial Insemination Center (RAIC) of South Sulawesi and evaluated in the In Vitro Embryo Production Laboratory, LPPM Building, Hasanuddin University, Makassar.

***Green tea extraction*:** Fresh tea leaves are washed and then dried in an oven at 100 °C. The dried tea leaves are ground with a blender and then sifted using a 70/100 mesh sieve. Extraction was performed following the procedure previously reported by Setyopratomo (2014).

***Semen collection and preparation*:** Semen from 3 Bali cows is collected using an artificial vagina. The semen collected from each cow is collected every two weeks in the morning. The artificial vagina that has been prepared is equipped with vaseline, hot water 150 mL at a temperature of 45 °C measured using a thermometer and a pump. Then, the collected semen is evaluated first; if it meets the SNI standards, it proceeds to the next stage with the addition of an Andromed diluent. Semen that meets the SNI standards is then divided into four parts. Each part is added with Andromed as a diluent that has been supplemented with GTP according to the treatment: T0: Semen + Diluent (Control), T1: Semen + Diluent + GTP 0.01 mg/100mL, T2: Semen + Diluent + GTP 0.02 mg/100mL, T3: Semen + Diluent + GTP 0.03 mg/100mL. Next, the semen is placed into mini straws (0.25 mL), equilibrated at 4°C for 2 hours, pre-frozen in liquid nitrogen vapor for 15 minutes, and stored in liquid nitrogen at -196°C.

***Semen and oocyte evaluation*:** The evaluation of spermatozoa motility is expressed as a percentage (Iskandar et al., 2022), with values ranging from 0-100% according to SNI (01-4869.1-2005) (Wittarja et al., 2020). According to Gustina et al. (2022), spermatozoa motility can be calculated using the formula:

$$\text{Motilitas} = \left(\frac{\text{Number of progressive spermatozoa}}{\text{Number of observed spermatozoa}}\right) \times 100\%$$

Spermatozoa viability is evaluated using eosin-nigrosin stain. Live spermatozoa are marked by a white head, while dead spermatozoa are marked by a redhead (Yendralize et al., 2019). The viability percentage is calculated using the formula based on Surahman et al. (2021):

$$A = \left[\frac{P}{P + Q}\right] \times 100\%$$

Description: A = Sperm viability percentage
P = The number of living spermatozoa
Q = The number of dead spermatozoa

Spermatozoa abnormalities are evaluated using eosin-nigrosin staining. Abnormalities are observed in spermatozoa that have abnormal shapes such as no sperm head, large head shape, broken tails, and coiled tails (Manehat et al., 2021). Spermatozoa abnormalities can be calculated using the formula (Yaramareddy et al., 2023):

$$TZI = (c + p + q) / x$$

Description: c = Head flaws, p = Midpiece flaws, q = Tail flaws, x = Total number of abnormal sperm

The evaluation of plasma membrane integrity (PMI) was conducted using the Hypoosmotic Swelling Test (HOS Test) based on the swollen and curled tail according to Revell and Mrode (1994). PMI is calculated using the formula (Diansyah et al., 2021):

$$I = \left[\frac{C}{C + S}\right] \times 100\%$$

Description: I = Plasma membrane integrity, C = The number of tails that are circling, S = The number of straight tails

Acrosome integrity is evaluated with a formalin-saline solution. Spermatozoa with an intact acrosome are marked by

1/2 to 2/3 of the anterior head being darker in color than the posterior part (Arifiantini et al., 2006). The percentage of intact acrosome can be calculated using the formula (Fatah et al., 2018):

$$\text{Acrosome Integrity} = \left(\frac{\sum \text{Intact Acrosome of Sperm}}{\sum \text{Total of Spermatozoa}}\right) \times 100\%$$

Evaluation of DNA fragmentation using acridine orange (AO) staining (Sigma-Aldrich®, St. Louis MO) and observed using a Z2 fluorescent imager microscope (Zeiss Company, Germany). DNA fragmentation was calculated using the formula (Adiputra et al., 2022):

$$\text{DNA Fragmentation Percentage} = \left(\frac{\text{Number of Damaged Spermatozoa}}{\text{Total Number of Spermatozoa}}\right) \times 100\%$$

Evaluation of H_2O_2 concentration in frozen semen and oocytes using 2', 7'-dichlorodihydrofluorescein diacetate (DCHF-DA, Sigma Co, USA) followed the procedure reported by Kiani-Eshafani et al. (2012). Fluorescence emission was recorded with a digital camera (Zeiss AxioCam HRc, Germany) on a fluorescence microscope (Zeiss Axio Imager A2). The obtained images were converted to TIFF files using Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA), and then analyzed with ImageJ 1.47 software (Sun Microsystems, Inc., California, USA). Fluorescent images were measured by counting the number of pixels after color inversion. The intensity of fluorescence represents the concentration of intracellular H_2O_2.

The IVF processes follow the procedures reported by Hasbi et al. (2020). Oocyte collection was performed using puncture technique. The oocytes used were Grade A and B (Damayanti et al., 2022). After maturation, the oocytes are washed in fertilization media twice, then placed into the next fertilization media, and incubated in an incubator (Thermo Fisher Scientific (USA)) with 5% CO_2 at a temperature of 38.5 °C for 24 hours. The evaluation of fertilization rates was conducted using 2% aceto orcein stain (Bhakty et al., 2021). The fertilization status is determined by the formation of 2 or more than 2 pronuclei.

***Statistical analysis*:** The research design used is a completely randomized design (CRD). Data was analyzed using analysis of variance (ANOVA) if there were differences between treatments, followed by Duncan's test using SPSS software ver. 25.

RESULTS

***Post-Thawed semen quality*:** The quality of Bali bull semen with the addition of GTP in the extender is presented in Table 1. Based on the results of the statistical analysis, the addition of GTP to the extender of Bali bull semen significantly affects viability, Acrosome integrity, plasma membrane integrity, DNA fragmentation, and concentration of H_2O_2 ($P<0.05$). It does not affect motility and sperm abnormalities ($P>0.05$).

***Fertilization Ability and H_2O_2 Concentration in Oocytes*:** The ability of sperm to fertilize oocytes is marked by the formation of 2 and >2 PN. The results of this study show that the number of oocytes forming 2 and >2 PN is significantly higher ($P<0.05$) in T2 compared to T0 and T3, but not different from T1 (Table 2).

DISCUSSION

Table 1. Quality and concentration of hydrogen peroxide (H_2O_2) in Bali bull semen with the addition of GTP in the diluent.

No.	Parameters	Treatments			
		T0	T1	T2	T3
1.	Motility (%)	60.00±0.00	60.00±0.00	60.00±0.00	60.00±0.00
2.	Viability (%)	70.38±2.57^{a}	78.45±6.24^{b}	80.35±5.01^{b}	75.50±4.81^{ab}
3.	Abnormalities (%)	15.93±1.11	16.17±2.88	15.16±2.90	14.44±2.03
4.	Acrosome integrity (%)	59.08±2.72^{a}	68.75±4.31^{b}	72.93±6.04^{b}	62.43±2.82^{ac}
5.	Plasma membrane integrity (%)	65.17±5.04^{a}	70.53±7.83^{a}	74.56±5.85^{b}	68.70±6.10^{a}
6.	DNA fragmentation (%)	5.95±1.00^{a}	4.34±0.75^{b}	3.03±0.92^{b}	3.80±1.02^{b}
7.	Concentration of H_2O_2 (FIU/spermatozoa)	5.04±1.33^{a}	2.82±1.75^{b}	2.12±0.67^{c}	3.08±1.58^{b}

^{abc} Different superscripts on the same line indicate a significant difference ($P<0.05$). T0 (Control), T1 (0.01 mg/100mL GTP), T2 (0.02 mg/100mL GTP) and T3 (0.03 mg/100mL GTP).

Table 2. Fertilization ability and H_2O_2 concentration of Bali cattle oocytes with GTP addition to spermatozoa.

No.	Parameters	Treatments			
		T0	T1	T2	T3
1.	2PN dan >2PN (%)	61.47±08.63^{a}	80.09±06.43^{bc}	85.99±13.57^{c}	70.91±10.19^{ab}
2.	H_2O_2 concentration (FIU/oocyte)	57.50±19.21^{a}	46.48±18.22^{b}	31.68±13.05^{c}	43.75±19.04^{b}

^{abc} Superscripts on the same line differ significantly ($P<0.05$). PN: pronucleus. T0 (Control), T1 (0.01 mg/100mL GTP), T2 (0.02 mg/100mL GTP), and T3 (0.03 mg/100mL GTP).

The addition of GTP does not have an impact on the motility of frozen Bali bull sperm. The concentration of GTP from 0.01-0.03 mg/100mL has not yet been effective in increasing the motility of Bali bull sperm. It is suspected that the polyphenols present in GTP do not provide antioxidant but pro-oxidant effects. However, they are still influenced by physiological conditions and the concentration of polyphenols that can activate Nrf2 (erythroid 2-related factor), which is considered the leading cause of antioxidant and cytoprotective effects in cell culture studies (Tyuryaeva & Lyublinskaya, 2023). The research results of Inanc et al. (2019) indicate that adding catechins derived from green tea extract does not increase the motility of bull sperm. Nevertheless, the sperm motility in this study is still suitable for artificial insemination (AI) because it meets the Indonesian National Standard (SNI), which has a minimum AI motility of more than 40%. The results of this study indicate that the administration of GTP at a concentration of T1 (78.45±6.24) and T2 (80.35±5.01) indicates significantly higher sperm viability (P<0.05) compared to T0 (70.38±2.57), but not different from T3 (75.50±4.81). In this case, GTP can increase the viability of Bali bull sperm and is more effective compared to the addition of 0.03 mg/100mL. These results align with the research by Pramesti & Ducha (2023), which found that adding green tea extract at a concentration of 3% increased the viability of Ongole Crossbred cattle spermatozoa after freezing. The same was reported by Susilowati et al. (2021) that the addition of green tea extract could increase the viability of Simmental bull sperm. The increase in sperm viability in this study is suspected to be due to the polyphenols in GTP, which have an antioxidant role because of their redox properties that can reduce lipid peroxidation in sperm (McNiven & Richardson, 2006). It has been reported by Chen et al. (2003) that polyphenols increase cell viability, reduce intracellular Ca^{2+} levels and ROS formation, and enhance mitochondrial membrane potential. Adding GTP up to a concentration of 0.03 mg/100mL does not affect the abnormality of Bali bull sperm but shows a tendency for a lower addition of 0.03 mg, with sperm abnormality around 14.44%. According to Ihsan (2009), mixing with diluents or during the preparation of rough preparations will increase damage to the spermatozoa head. However, the semen in this study can still be used for Artificial Insemination. According to Sekosi et al. (2016), semen for Artificial Insemination purposes should not contain more than 20% abnormal sperm.

The percentage of acrosome integrity with adding GTP at a concentration of T1 (68.75±4.31) and T2 (72.93±6.04) are significantly higher (P<0.05) compared to T0 (59.08±2.72) and T3 (62.43±2.82) where T2 is significantly higher compared to the other treatments. The results of this study are in line with the research by El-Sheshtawy et al. (2021) that the addition of green tea extract to egg-yolk citrate diluent resulted in an acrosome integrity percentage of Bali bull sperm of 78.19%, which is higher compared to without the addition of GTD This indicates that polyphenols can act as antioxidants and are capable of protecting the integrity of the acrosome and preventing premature acrosome reactions (Qamar et al., 2023). The acrosome integrity has a quite important function for the success of fertilization (He et al., 2022). This is related to the enzymes contained within it (Khawar et al., 2019). Damage to acrosome integrity will cause enzymes to be released, leading to the loss of spermatozoa's ability during fertilization (Stival et al., 2016; Sun et al., 2020).

The percentage of plasma membrane integrity from the research results shows the highest value in T2 (74.56±5.85) is significantly higher (P<0.05) compared to T0 (65.17±5.04), T1 (70.53±7.83), and T3 (68.70±6.10). GTP in semen diluents has a positive effect on the plasma membrane integrity of Bali bull semen. A coiled tail marks sperm with intact plasma membranes, whereas a straight tail marks those with damaged membranes. Khan et al. (2017) reported that green tea contains polyphenol compounds that can be used as antioxidants capable of suppressing peroxidation effects, thereby inhibiting damage to frozen semen membranes. Furthermore, Prastiya et al. (2023) reported that adding green tea extract significantly affected the proportion of spermatozoa with intact plasma membranes during pre-freezing. The same was reported by Khattak et al. (2022) that the addition of green tea extract had a positive impact on the intact plasma membrane of buffalo spermatozoa post-thawing.

The research results showed that adding GTP can significantly reduce DNA fragmentation levels in Bali bull semen significantly lower (P<0.05) in T1 (4.34±0.75), T2 (3.03±0.92), and T3 (3.80±1.02) compared to T0 (5.95±1.00), where T2 is significantly lower than others. DNA fragmentation in sperm can be marked by the orange-colored head of the spermatozoa stained with acridine orange. Mustofa et al. (2021) reported that adding green tea extract at a 0.1 mg/100mL concentration reduced DNA fragmentation in post-thawing Kacang goat spermatozoa. In addition to potentially causing DNA damage, high concentrations of ROS can also affect motility, morphology, and lipid peroxidation in sperm (Ko et al., 2014). The results of this study indicate that the polyphenols contained in GTP can suppress DNA damage in spermatozoa. Polyphenols can interact with spermatozoa components and reduce lipid peroxidation caused by free radicals (Hyon, 2004). The research results show that adding GTP can reduce the concentration of sperm H_2O_2, where T2 (2.12±0.67) is significantly lower compared to T0 (5.04±1.33), T1 (2.82±1.75) and T3 (3.08±1.58). This shows that the polyphenols contained in GTP benefit Bali bull spermatozoa. Polyphenols react with ROS to form relatively stable phenolic oxygen radicals, thereby eliminating free radicals by increasing the activity of glutathione peroxidase and

superoxide dismutase, and their cleansing power is much stronger than that of vitamins C and E. This is due to active hydroxyl hydrogen groups in the polyphenol molecular structure in GTP, which can terminate excessive free radical chain reactions (Bhutia et al., 2015). The concentration of H_2O_2 is observed based on the intensity of light formed with DCHF-DA staining; the brighter the color formed, the higher the concentration of H_2O_2, and vice versa. This can occur because the high production of ROS exceeds the capacity of antioxidants. ROS includes Superoxide Radicals (O_2•−), Hydrogen Peroxide (H_2O_2), and Hydroxyl Radicals (•OH) in the cryopreservation process (Huang et al., 2018). H_2O_2 is a ROS that can diffuse from its production site in the midpiece of sperm to the head (Aitken et al., 2012). GTP added to the spermatozoa diluent showed a significantly higher number of oocytes forming 2 and >2 PN ($P<0.05$) at T2 (85.99±13.57) compared to T0 (61.47±8.63) and T3 (70.91±10.19), but not different from T1 (80.09±6.43). With the addition of 0.02 mg/100 mL, the best fertilization ability can reach 85.99%. This shows that GTP added to the diluent as an antioxidant can enhance the sperm's ability to fertilize oocytes in vitro. Normal fertilization is defined by the presence of 2 pronuclei (2 PN) and fertilization by 2 sperm, resulting in the formation of 3 pronuclei (3 PN), which is also used to describe the occurrence of the fertilization process (Wun et al., 2020). The addition of GTP in the diluent is suspected to protect against membrane damage and maintain the integrity of the sperm acrosome due to high ROS production, thereby not interfering with sperm-oocyte fusion. Tremellen (2008) reported that high levels of ROS and oxidative stress have been associated with the pathophysiology of infertility. High levels of ROS (hydroxyl superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite) are detrimental to sperm motility, viability, and function, interacting with membrane lipids, proteins, nuclear, and mitochondrial DNA (Anghel et al., 2010). The addition of GTP to sperm can reduce the concentration of H_2O_2 in the in vitro fertilized oocytes, where T2 (31.68±13.05) was significantly lower ($P<0.05$) compared to T0 (57.50±19.21), T1 (46.48±18.22), and T3 (43.75±19.04). The concentration of H_2O_2 in the oocyte can be observed based on its light intensity. This indicates that adding GTP 0.02 mg/100 mL can prevent the formation of free radicals in Bali cow oocytes resulting from *in vitro* fertilization. According to the study by De Castro et al. (2016), it was verified that sperm introduced into an oxidative environment negatively affects embryo development during in vitro fertilization. Iskandar et al. (2019) reported that one of the factors that also affects *in vitro* fertilization ability is ROS production. Dead spermatozoa produce ROS that cause membrane lipid peroxidation, reducing membrane fluidity and sperm function. High levels of ROS damage spermatozoa metabolism in in vitro fertilization media.

***Conclusion*:** It is concluded that the addition of GTP to the extender has positive implications by improving sperm quality, namely viability, acrosome integrity, plasma membrane integrity, reducing DNA fragmentation in Bali cattle, improving in vitro fertilization and lowering the concentration of H_2O_2 in sperm and oocytes resulting from fertilization. The best GTP concentration in the Bali bull extender in this study is 0.02 mg/100mL.

***CRediT author statement*:** Yuni Sartika, the conceptualization of the manuscript, data collection, and draft writing; Herry Sonjaya contributes to monitoring and review of the manuscript; Sri Gustina contributes to the review of the IVF process; Erni Damayanti contributes to creating the collection and fertilization media; Hasbi Hasbi contributes to methodology, review, and data accuracy. All authors have read the paper.

***Conflict of interest*:** The authors declare no conflict of interest.

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***Availability of data and materials*:** We declare that the submitted manuscript is our original work, which has not been previously published and is not being considered for publication elsewhere.

Consent for publication: All authors have submitted consent to publish this research article in JGIAS.

***SDG's addressed*:** Quality Education, Good Health and Well-being, Zero Hunger

***Policy referred*:** Indonesia's national livestock improvement and breeding policy; Improve the productivity and genetic quality of local cattle breeds (such as Bali cattle) through artificial insemination (AI); Promote the use of natural antioxidants and biotechnological innovation.

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